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Model substrates for cutinases

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Abstract

As a part of our study of microbial cutinases, a mixture of oligomers of 16-hydroxyhexadecanoic acid has been prepared by partial polymerization to serve as defined substrates. The mixture has been characterized by spectroscopic and chromatographic methods. Enzyme-catalyzed hydrolysis of the oligomers using fungal lipase, fungal cutinase or bacterial lipase required the addition of β-cyclodextrin to the reaction medium, whereas apple and other naturally occurring cutins can be enzymatically hydrolyzed without the aid of this cyclic oligosaccharide. The difference in reactivity may be due to the greater hydrophobicity of the synthetic oligomers compared with natural cutin polymers.

Key words: Cutin; Esterases; Hydroxyhexadecanoate; Polyester; Cyclodextrin

1. Introduction

Cutin, a very hydrophobic, insoluble polyester composed of ω-hydroxy fatty acid derivatives, is the structural component of the plant cuticle. The polymer is cross-linked through either hydroxy or epoxy substituents in the fatty acid chain [1]. The structure of the cross-links has yet to be characterized. A structure for cutin has been proposed based on gas chromatographic mass spectral (GC/MS) analysis of the monomers obtained from depolymerized cutin [2]. The cutin polymer structure has also recently been examined by NMR [3,4]; however, a detailed structure of this important biopolymer is still incomplete. Enzymatic degradation of cutin offers another means of probing the cutin structure. Our laboratory has recently examined a number of bacterial species for cutinase activity [5,6]. The cutinase activity of these bacteria was assayed using cutin derived from apples as the substrate. In conjunction with this research we have synthesized a series of oligomers of 16-hydroxyhexadecanoic acid, a monomer that is a common constituent of many cutins, as model substrates for studying the mechanism of cutinase depolymerization of cutin. The synthesis, separation and characterization of

oligomers of 16-hydroxyhexadecanoic acid are the subjects of this report. Initial results of the reaction of these substrates, which were catalyzed by a well characterized fungal cutinase and lipases, will also be described.

2. Experimental

2.1. Oligomer synthesis

Approximately 10 mg of *p*-toluenesulfonic acid was added to a solution of 3.5 g of 16-hydroxyhexadecanoic acid in 28 ml of benzene and refluxed for 3 h in a 50 ml round-bottom flask fitted with a Dean-Stark trap and condenser (after 2 h no more water appeared to be collecting in the trap). The precipitate that formed on cooling was filtered, washed with benzene and diethyl ether and dried under vacuum (yield, 3 g).

2.2. Oligomer separation and analysis

TLC of the oligomer mixture was carried out on 10 cm × 10 cm silica gel HP-K (Whatman) plates using $CHCl_3$:MeOH (95:5) as the mobile phase. The plates were sprayed with a solution of 10% $CuSO_4 \cdot 5H_2O$ in 8% aqueous phosphoric acid and then heated on a hot plate for visualization.

Open column chromatography was carried out on a silica gel (Woelm) column (2 × 45 cm) eluted with $CHCl_3$:MeOH (95:5); 5 ml fractions were collected and monitored by TLC as described above.

2.3. Oligomer characterization

Oligomer fractions were hydrolyzed in 1 N NaOH at 75°C for 16 h. The hydrolysate was extracted with $CHCl_3$ after neutralization with 1 N HCl and then converted to the TMS methyl ester derivative with diazomethane followed by silylation with BSA reagent (Alltech).

The TMS ester was analyzed on a Hewlett-Packard 5995B GC/MS fitted with a 10 M Ultra capillary column (Hewlett-Packard); the column temperature was programmed from 125 to 250°C at 4°/min. FAB-MS of the cutin oligomer mixture was obtained on a VG Analytical ZAB 2-SE high-field mass spectrometer at an accelerating voltage of 8 kV in the negative ion mode; the matrix for the sample was *m*-nitrobenzyl alcohol. ^{13}C-NMR spectra (without NOE) of $CDCl_3$ solutions of oligomers were obtained on a JEOL 400 spectrometer at 100 mHz with a pulse width of 8 μs (~60°), a pulse delay of 1 s and a broadening factor of 3 Hz.

Table 1
Reaction media for enzymatic hydrolysis of synthetic cutin oligomers

Enzyme	Adjuvant	Adj (%)	Solvent[a]	Hydrolysis
Lipase PS-800	—		Buffer	–
Pseud.[b] lipase	—		Buffer	–
Fus.[c] cutinase	—		Buffer	–
Lipase PS-800	—		Buffer/$CHCl_3$	–
Pseud. lipase	—		Buffer/$CHCl_3$	–
Fus. cutinase	—		Buffer/$CHCl_3$	–
Lipase PS-800	*n*-octyl glucoside	1	Buffer	–
Fus. cutinase	*n*-octyl glucoside	0.1	Buffer	–
Lipase PS-800	Na^+ deoxycholate	1	Buffer	–
Fus. cutinase	Na^+ deoxycholate	0.1	Buffer	–
Lipase PS-800	β-cyclodextrin	2.5	Buffer	+
Pseud. lipase	β-cyclodextrin	2.5	Buffer	+
Fus. cutinase	β-cyclodextrin	2.5	Buffer	+
—	β-cyclodextrin	2.5	Buffer	–

[a]For reaction conditions see experimental.
[b]Pseud.: *Pseudomonas.*
[c]Fus.: *Fusarium solani* f sp. *pisi.*

2.4. Enzyme hydroysis of oligomers

For each reaction, 30 mg of unfractionated, monomer-free oligomers was suspended in 2 ml of 0.1 M buffer. Enzymatic hydrolyses were carried out for 40 h at 40°C with shaking with (a) 20 mg of PS-800 lipase (Amano) in potassium phosphate buffer, pH 8, (b) 8 mg of *Pseudomonas* lipase (Sigma) in potassium phosphate buffer pH 8 and (c) 10 mg of cutinase isolated in our laboratory from *Nectria haematoccoca* (*Fusarium solani* f. sp. *pisi*) in glycine buffer pH 9. A summary of the incubation variables is given in Table 1. Solutions were then extracted with an equal volume of $CHCl_3$, the $CHCl_3$ solution concentrated to dryness and the residue derivatized as above and analyzed by GC/MS.

3. Results and discussion

The products of partial polymerization of 16-hydroxyhexadecanoic acid were most effectively separated by TLC on Whatman high performance plates (HP-K) (Fig. 1). At least 16 distinct spots were observed on TLC, which indicated that oligomers of degree of polymerization (DP) 16 or greater were produced under the described polymerization conditions. Attempts to prepare pure oligomers by column chromatography, preparative HPLC (on reverse and normal phase columns using a variety of solvent systems) or preparative TLC were unsuccessful. The best purification was obtained by column chromatography in conjunction with preparative TLC (analytical plates), which gave a series of oligomer fractions that, in most cases, contained just two oligomers. Base hydrolysis of these fractions yielded 16-hydroxyhexadecanoic acid as the only product.

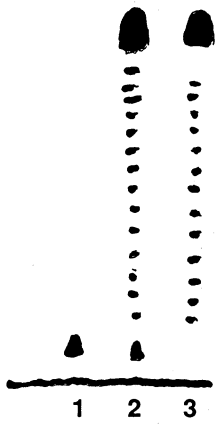

Fig. 1. TLC of 1,16-hydroxyhexadecanoic acid; mixture of 16-hydroxyhexadecanoic acid and synthetic oligomers; 3, synthetic oligomers.

The ^{13}C-NMR spectrum and the FAB-MS analysis of the unfractionated oligomer mixture were consistent with the polyester composition of the mixture. The negative ion FAB-MS (Fig. 2) shows ions corresponding to oligomers up to DP 6 (Fig. 2; DP 2, [M-H] = 525; DP 3, [M] = 780; DP 4, [M] = 1034; DP 5, [M] = 1288; DP 6, [M] = 1542). Ions corresponding to higher molecular weight oligomers were not observed, because of their low concentration in the oligomer mixture (Fig. 1) and instrumental limitations.

One major resonance (174 ppm) was observed in the downfield region of the NMR spectrum as well as a minor peak at 177.6 ppm, which correspond to ester and acid carboxyl carbon, respectively. On the basis of carbonyl peak areas (assuming the same relaxation times for the ester and acid carbons) the average DP of the oligomer mixture was determined to be 15. This value was consistent with that obtained by comparing α-methylene carbon resonances of the terminal acid and internal ester.

The unfractionated oligomer mixture was used for initial enzyme studies because of the difficulty in preparing pure oligomers (other methods of purification are being explored), particularly in amounts necessary for the enzyme assay.

In contrast to apple cutin, the synthetic oligomer mixture is more difficult to hydrolyze either enzymatically with PS-800 lipase, *Pseudomonas* lipase or *Nectria* cutinase or chemically, even though both substrates are water-insoluble. It appeared that the synthetic mixture composed of unsubstituted oligomers is much more hydrophobic than naturally occurring cutin, which may account for this difference in reactivity. Enzymatic hydrolysis of the synthetic cutin oligomer mixture could not be achieved in H_2O, in a bisolvent system ($H_2O/CHCl_3$) or with the addition of detergents (Table 1). Enzymatic hydrolysis did

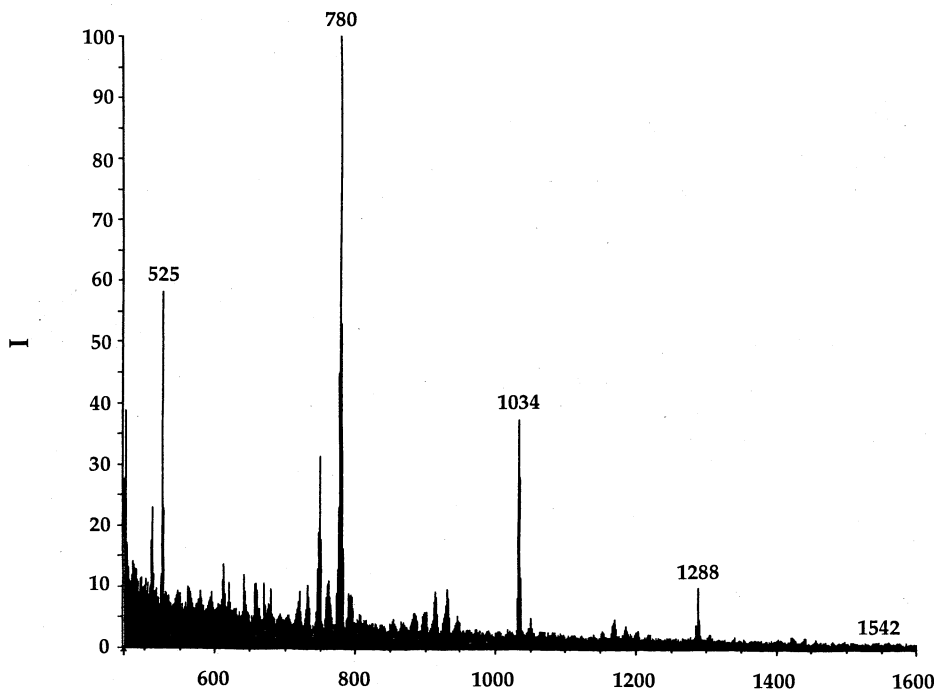

Fig. 2. Negative ion FABS-MS of synthetic oligomer mixture.

occur when the reaction was run in the presence of β-cyclodextrin. Cyclodextrins (cyclic glucans; β-cyclodextrin having a DP = 7) have been used to promote ester hydrolysis [7] and in lipase hydrolysis of triglycerides [8]. A noticeable increase in the wetability (i.e., water adhering to the polymer) of the cutin oligomers was apparent when β-cyclodextrin was incorporated into the reaction mixture. Enzymatic hydrolysis of apple cutin also occurred to a greater extent in the presence of β-cyclodextrin (unpublished results), A further examination of the applicability of cyclodextrins as adjuvants for enzymatic hydrolysis of cutin and the mechanism of β-cyclodextrin-assisted hydrolysis is in progress.

Although β-cyclodextrin is required for the enzymatic hydrolysis of synthetic cutin oligomers, these oligomers should still be useful as probes for studying cutinase activity. In the future it is hoped that sufficient quantities of pure oligomers can be isolated to study the effect of such variables as substrate chain length on enzyme activity and the mode of hydrolysis (i.e. endo or exo) of the enzyme. The preparation of substituted cutin oligomers (e.g. oligomers resulting from the polymerization of 9,18-dihydroxyoctadecanoic acid where one of the hydroxyls has been blocked to prevent crosslinking) is in progress.

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